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Ranque–Hilsch vortex tube thermocycler for fast DNA amplification and real-time optical detection

Ryan J. Ebmeier

University of Nebraska-Lincoln

Scott E. Whitney

Department of Chemical and Biomolecular Engineering

Amitabha Sarkar

Department of Chemical and Biomolecular Engineering, amitabhas@yahoo.com

Michael Nelson

Megabase Research Products, Lincoln, Nebraska

Nisha Padhye

Megabase Research Products, Lincoln, Nebraska, npadhye2@unlnotes.unl.edu

See next page for additional authors

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Authors

Ryan J. Ebmeier, Scott E. Whitney, Amitabha Sarkar, Michael Nelson, Nisha Padhye, George Gogos, and Hendrik J. Viljoen

Ranque–Hilsch vortex tube thermocycler for fast DNA amplification and real-time optical detection

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An innovative polymerase chain reaction (PCR) thermocycler capable of performing real-time optical detection is described below. This device utilizes the Ranque–Hilsch vortex tube in a system to efficiently and rapidly cycle three 20 mL samples between the denaturation, annealing, and elongation temperatures. The reaction progress is displayed real-time by measuring the size of a fluorescent signal emitted by SYBR green/double-stranded DNA complexes. This device can produce significant reaction yields with very small amounts of initial DNA, for example, it can amplify 0.25 fg (5 copies) of a 96 bp bacteriophage λ -DNA fragment 2.731011-fold by performing 45 cycles in less than 12 min. The optical threshold (150% of the baseline intensity) was passed 8 min into the reaction at cycle 34. Besides direct applications, the speed and sensitivity of this device enables it to be used as a scientific instrument for basic studies such as PCR assembly and polymerase kinetics.

I. INTRODUCTION

The polymerase chain reaction (PCR) is a powerful and sensitive enzymatic technique used to exponentially increase the number of copies of a specific sequence of template DNA. The PCR process includes three steps: sample preparation, DNA amplification, and detection. After preparation, the sample mixture (includes DNA, primers, and polymerase) is allotted into a desirable sample size (typically 1–100 μ L for amplification). The amplification step consists of thermally cycling the sample for N cycles (~ 35) between specific denaturation, annealing, and elongation temperatures; typically 90–95°, 50–65°, and, 72 °C, respectively. Theoretically, one cycle will double the existing amount of DNA, but in practice the PCR process can be described in terms of overall efficiency s_Y , and

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where $X = (1 + Y_d)^N$ is the DNA amplification yield. Typical PCR amplification has an efficiency of 70% and requires 35 cycles for 108-fold amplification. Gel electrophoresis is the standard for product detection. However, real-time detection can be achieved by measuring the fluorescence of dye/DNA complexes throughout the amplification stage of the reaction. This drastically reduces total assay time by eliminating the need for an individual step for product detection. Real-time PCR also provides the required optical measurements used in quantitative PCR analysis. There are many types of PCR machines used in practical applications. However, these devices may generally be classified into two categories: robotic devices which move the DNA samples to the heat; and thermocyclers which bring the heat to the samples. Robotic devices, such as Stratagene's ROBOCYCLER™ (Stratagene, La Jolla, CA), move tubes containing PCR reaction samples to and from a series of water baths, which are heated to different temperatures. Although robotic thermocyclers can be useful in certain applications, they are incapable of high-speed PCR due to their rates of heating and cooling (2 min/ cycle). The two basic types of thermocyclers are programmable heat blocks and forced hot-air thermocyclers. In commonly employed heat block thermocyclers, the amplification stage consists of cycling the temperature of the samples using computer controlled heat blocks. These devices typically require hours of operation time due to the slow heating and cooling processes. Forced hot air thermocyclers, such as the LightCycler™ (Roche Diagnostics Corporation, Germany) have drastically reduced typical amplification time by eliminating the large thermal mass of heat blocks and utilizing convection heat transfer between air and thin walled capillary tubes. PCR amplification consisting of 30 cycles can be performed in as little as 10–30 min. Some of the most recent publications describe PCR results using natural convection processes. For example,

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Krishnan et al.¹ used a Rayleigh-Benard convection cell to amplify 315 ng of DNA in a 35 mL sample with a reaction time of 1.5 h. The amplification and reaction time reported is comparable to standard PCR thermocyclers. Braun et al.² amplified 500 pg of bacteriophage I-DNA in a 20 mL sample 105-fold within 25 min of assay time. The heating and cooling performance is the primary concern for all automated thermocyclers. The innovation of the thermocycler described in this article relies on taking advantage of the natural heating and refrigeration capacities of the Ranque–Hilsch vortex tube. A vortex tube is a device that produces two gas streams of significant temperature difference from a single source of compressed gas. An attractive feature of the vortex tube is its reliability—it operates without any moving parts or electrical components. The inlet gas pressure is typically in the range of two to eleven atmospheres and enters a cylindrical container tangentially through one or more ports. After entering the cylinder, the gas spirals down the length of the tube (see Fig. 1) where it meets a control valve. According to the valve setting, a fraction of the gas s_2 will exit the tube at an outlet temperature up to about 110 °C warmer than the inlet temperature of the gas. The remaining gas fraction s_1 exits at the other end with a maximum temperature difference of about 70 °C lower than the initial gas temperature. The outlet temperatures can be varied according to the absolute pressure drops across the tube, the type of gas used, and with the control valve that changes the fraction of gas exiting at each end. Figure 1 illustrates vortex tube operation for one set of flow conditions. The vortex tube was discovered by Ranque³ and a systematic explanation of its performance was achieved by Hilsch.⁴ However, since that time there has been dispute over the numerous theoretical and experimental approaches used to describe the physical aspects of the

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vortex tube, now called the Ranque–Hilsch effect. More recently, these explanations vary from the effects of acoustic streaming in swirling flow researched by Kurosaka⁵ to the characterization of the vortex tube as a natural heat pump due to a secondary circulation.⁶ Ahlborn and Gordon⁷ have quantified the performance of the vortex tube as a thermodynamic machine.

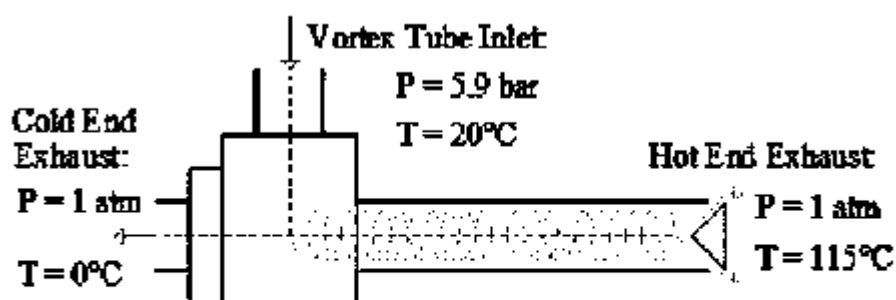


FIG. 1. (Color online) Ranque–Hilsch vortex tube conditions for basic thermocycler operation.

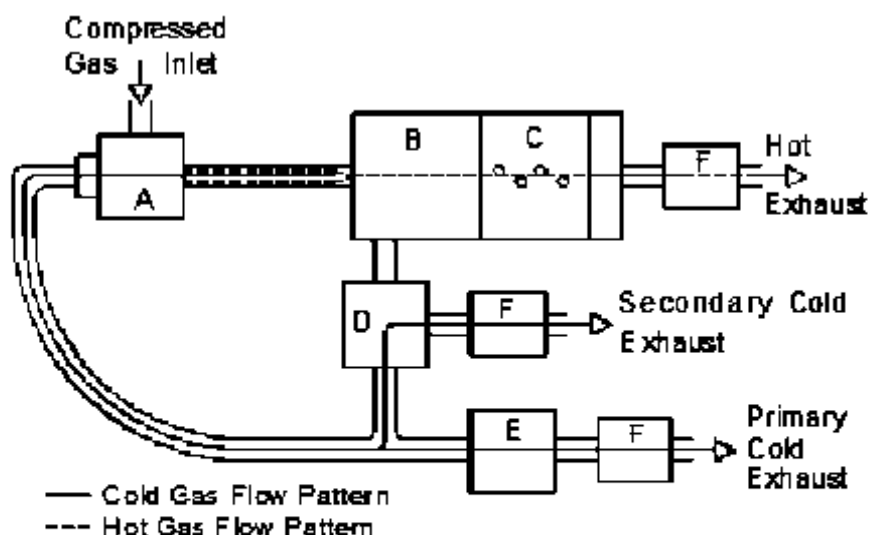


FIG. 2. (Color online) Flow pattern for sample heating: (A) Ranque–Hilsch vortex tube; (B) vortex flow conditioner; (C) sample chamber; (D) cold flow valve; (E) cold release valve; (F) exhaust muffler.

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II. RANQUE–HILSCH VORTEX THERMOCYCLER

The majority of vortex tube applications are associated with the tube's refrigeration capacity. The Ranque–Hilsch vortex tube thermocycler presented in this article uses a vortex tube within a system. Computer control and pneumatic flow valves are employed to use both the hot and cold exhaust streams as necessary to heat/cool the PCR samples. The compact design of the thermocycler gives it overall dimensions of 12 in.×38 in.×36 in. It also has very limited dependence on electricity and can be controlled using a laptop computer. The entire device is based on the simplicity and reliability of the vortex tube and effectively cycles three, 2 mL, sample capillaries within the range of PCR typical temperatures. The device is entirely dependent on the performance of the vortex tube (Exair Inc., Cincinnati, OH), so its operational parameters were optimized. The compressed air supply used is capable of delivering room temperature air at a flow rate of 3.23×10⁻³ m³/s (6.8 SCFM) at a gage pressure of 5.9 bar (85 psid). The first step in optimizing the vortex tube is to size the generator, a flow restriction device, to correspond with the available air supply. The appropriate generator should maximize the volumetric flow rate, while providing enough resistance to create a large pressure drop across the tube. The tube operates most efficiently at inlet pressures of about 5.5–7.5 bar (80–110 psid). The exit temperatures are variable according to the ratio of gas exiting at each end. Typically, the exit temperature difference at each end is inversely related to the mass fraction of gas exiting. For this application, experimentation concluded that the hot gas temperature must exceed 100 °C to ensure the sample can still reach the denaturation temperature after experiencing minimal heat loss throughout the system. To obtain the minimum

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increase in hot gas temperature of 80 °C, the vortex tube must be set so the fraction of hot gas exiting is low, ,20% of the inlet flow. The resulting cold flow exits the vortex tube at about 0 °C. Accordingly, the tube becomes more effective during the cooling process and sample heating becomes the rate-limiting step. The setup of the thermal cycling device is designed to optimize the convective heat transfer to the sample capillaries during both the heating and cooling processes (Fig. 2). The temperatures of the PCR samples are indirectly measured via a thermal sensor placed in an additional capillary tube. This signal is continually recognized within the computer programming, which in turn controls the status of two electronically actuated pneumatic valves. Both of these valves are used to direct the cold air flow within the system according to the sample temperature change required. The system heats the samples by only allowing the hot air to pass through the sample chamber as shown in Fig. 2. The three-way cold flow valve is closed, while the two-way FIG. 1. (Color online) Ranque–Hilsch vortex tube conditions for basic thermocycler operation. FIG. 2. (Color online) Flow pattern for sample heating: (A) Ranque–Hilsch vortex tube; (B) vortex flow conditioner; (C) sample chamber; (D) cold flow valve; (E) cold release valve; (F) exhaust muffler. Rev. Sci. Instrum., Vol. 75, No. 12, December 2004 Ranque-Hilsch vortex tube thermocycler 5357

cold release valve is open. This valve configuration provides two exhausts for all of the cold air produced by the vortex tube to exit the system with negligible back pressure. Meanwhile, the air exiting the hot end of the vortex tube flows across the samples located in the sample chamber before exiting the system via the sample chamber exhaust. The vortex tube is adjusted so the hot gas temperature is ,115 °C and the corresponding volumetric flow rate of hot gas produced is ,6.4310–4 m³/s (1.4 SCFM). The machine can heat the samples at an average rate of ,3.2 °C/s. The transition

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from heating to cooling must be performed without interrupting the operating vortex tube by stopping flow on the cold side. Therefore, the cold flow valve is opened before closing the cold release valve. Adequate cooling is achieved under the configuration where all of the cold and hot gases mix in the vortex flow conditioner before reaching the sample chamber. Since the mass flow rate of cold gas is about four times greater than the hot gas, it is overwhelming and the resulting stream works to cool the samples to the annealing temperature. The cold gas is -5°C before reaching the two valves and the cold/hot gas mixture is typically around 30°C . The samples are cooled at an average rate of 16°C/s . When a temperature hold is included in a PCR protocol, the programming logic uses set temperature limits and temperature versus time slope measurements to control the status of the cold flow valve and keeps the sample chamber at the desired temperature. In many cases, holds are not necessary at the denaturation and elongation temperatures. Under these circumstances, overshooting, and undershooting is minimized through feedback control from the previous thermal cycle. This enables the machine to make heating/cooling transition adjustments within a PCR amplification to maximize performance by accurately reaching the desired target temperatures. Another important factor is the temperature deviation between the samples. The vortex flow conditioner is the first step in minimizing sample temperature discrepancy by effectively mixing the hot and cold gases to provide nearly isothermal inlet flow conditions. The arrangement of sample capillaries then becomes the primary concern. The staggered arrangement (Fig. 2) reduces the effect of the first capillary shadowing the subsequent ones. Also, the sample spacing is minimized to reduce the effect of heat loss to the chamber walls. The thermal sensor is placed in the downstream capillary position to ensure the other three capillaries have reached the desired temperatures. The temperature variance is

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most important at the denaturation and annealing temperatures. When these are set at 90 and 56 °C, respectively, the maximum temperature a sample reaches is 91.5 °C while the minimum is 55 °C. The other capillaries are between these limits and the desired temperature. The real-time detection is conducted by monitoring the fluorescence in one of the capillaries as the reaction progresses. The reading is taken once per cycle during the heat to denaturation step at a specified read temperature. When the capillary reaches the user designated temperature a blue light source (LS450, Ocean Optics, Dunedin, FL) shines light at 470 nm into the capillary where it reacts with the dye/double stranded DNA complexes. The fluorescence emitted by these complexes is then recorded by a CCD array based spectrometer (USB2000, Ocean Optics) designed for operation within the VIS/NIR range of wavelengths, 370–1050 nm.

III. EXPERIMENTAL PROCEDURE

The performance of the Ranque–Hilsch vortex tube thermocycler is demonstrated using a 96 bp I-DNA amplicon. The sensitivity of the machine and the ability to monitor a PCR reaction real-time was tested by varying the initial DNA concentrations from 0.25 to 100 fg. Reaction progress was monitored by measuring the fluorescence emitted at 520 nm by SYBR Green bound to double-stranded DNA complexes upon excitation at 470 nm. This dye binds to all double-stranded DNA and cannot distinguish between a specific PCR amplicon and primer-dimer artifact. Therefore, careful design of primers, optimum reaction conditions, and a hot-start PCR technique is very crucial

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when using SYBR Green for real-time PCR. The oligonucleotide primers described by Braun et al.² were used to amplify the target λ-DNA fragment. The primer sequences were 5'– GAT-GAGTTCGTGTCCGTACAACCTGG–3' and 5'– GGGCAATCAGTTCATCTTTCGTCATGG–3' with melting temperatures of 61 and 62 °C, respectively. The reaction mixture consisted of 600 nmol of each primer, 100–0.25 fg of λ-DNA (Fermentas Inc., Hanover, MD), 5 mM magnesium sulfate, 200 mM dNTPs, 13 SYBR Green I (S7563, Molecular Probes), 600 mg/mL BSA, and 0.8 unit of KOD hot start polymerase (Toyobo Co. Ltd., Japan) in 13 KOD hot start polymerase buffer. The speed of the Ranque–Hilsch vortex tube thermocycler is best utilized in combination with a fast enzyme, such as KOD hot start polymerase. This enzyme requires an initial 30 s hot start exceeding 90 °C to become fully activated. During this period, thermolabile antibodies bound to the DNA polymerase are inactivated, as described by Mizuguchi et al.⁸ Therefore, each reaction consisted of initial activation of the enzyme at 95 °C for 30 s, followed by 45 cycles of 95 °C for 0 s, 62 °C for 0 s, and 72 °C for 0 s. There are no holding times required for the denaturation and annealing steps since they happen almost instantaneously. Elongation occurs within a temperature range surrounding 72 °C. Therefore, a large portion of the ramp time from the annealing to the denaturation temperature is utilized for elongation. If a fast enzyme such as KOD polymerase is used (300 nt/s at 72 °C), it is possible to copy up to 1000 bp during the heating stroke, without pausing at 72 °C. The amplification consisted of 45 cycles to demonstrate machine sensitivity to 0.25 fg of bacteriophage λ-DNA (5 copies). Photon counting was carried out once per cycle using a 500 ms integration time when the sample reaches 80 °C while heating to the denaturation temperature. Samples were also analyzed by 2.5% agarose gel electrophoresis in TAE buffer. The DNA fragments were visualized

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using 312 nm UV light and photographed with Polaroid Type 667 film. 5358 Rev. Sci. Instrum., Vol. 75, No. 12, December 2004 Ebmeier et al.

IV. RESULTS AND DISCUSSION

The total reaction time, which includes the 30 s hot start and subsequent 45 cycles between the denaturation, annealing, and elongation temperatures, was less than 12 min. Figure 3 shows the temperature versus time profile for the hot start and initial three cycles of the PCR reaction. Figure 4 shows that positive results were obtained for each initial concentration of I-DNA while the negative controls (no initial I-DNA) remained negative. This gel picture was used to conduct amplification and efficiency calculations to demonstrate machine sensitivity. An initial DNA amount of 0.25 fg produced an amplification of 2.7310^{11} - fold, which corresponds to an overall efficiency of 79.5%. The corresponding real-time PCR spectrometer data are shown in Fig. 5. The PCR reaction is deemed successful when the intensity increases beyond an established threshold value defined as 150% of the baseline intensity. The number of cycles necessary to reach the threshold value increases as the initial DNA concentration is reduced. In the absence of primer–dimer artifacts, the negative control should not exceed the threshold value. Quantitative PCR is possible by calculating the initial concentration of DNA using the number of cycles necessary for the intensity to surpass the threshold value. The 0.25 fg DNA sample surpassed the threshold value 8 min into the reaction at cycle number 34. Concluding, the Ranque–Hilsch vortex tube thermocycler is capable of performing rapid

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PCR with real-time optical detection. It produces significant reaction yields with very small amounts of initial DNA. Positive results have been obtained from numerous tests amplifying a wide variety of DNA sources. Fluorescence data obtained throughout the reaction minimizes total assay time and can be used for quantitative PCR. Besides direct applications, the speed and sensitivity of this device enables it to be used as a scientific instrument for basic studies such as PCR assembly and polymerase kinetics.

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FIG. 3. Temperature vs time profile for PCR reaction: includes 30 s hot start followed by initial three cycles of the total 45 cycles programmed for 95, 62, and 72 °C without temperature holds at these temperatures.

FIG. 4. PCR amplification of 0–100 fg of 96 bp I-DNA amplicon. (MW) 100 bp molecular weight marker, (1 and 2) negative control, (3 and 4) 0.25 fg DNA, (5 and 6) 1 fg DNA, (7 and 8) 10 fg DNA, (9 and 10) 100 fg DNA.

FIG. 5. (Color online) Spectrometer intensity vs cycle number for increasing initial DNA concentrations. Rev. Sci. Instrum., Vol. 75, No. 12, December 2004 Ranque-Hilsch vortex tube thermocycler 5359